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MECHANISM OF ACTION OF TETANUS TOXIN

ANNUAL\FINAL REPORT

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We have previously reported that tetanus toxin (TT) increased ATP-dependent Ca++ uptake by human neutrophil lysosomes, and we hypothesized that the toxin activates the Ca++ ATPase present in these organelles. However, we have been unable to show any phosphorylating effect of TT on any lysosomal protein. In subsequent experiments, using mitochondrial inhibitors and a mitochondrial substrate, we showed that the stimulation of ATP-dependent Ca++ uptake is an effect of TT on mitochondria. We confirmed this finding by using purified bovine adrenal mitochondria. However, we were then unable to show any specificity for the TT action by using TT-specific ganglioside affinity chromatography, as well as immunoabsorption with monoclonal antibodies. We conclude that the described TT effect was due to the presence of phosphate salts in the TT preparation which facilitated Ca++ transport by mitochondria.

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In the previous annual report, we described our finding that tetanus toxin (TT) irreversibly stimulates ATP-dependent Ca^{++} uptake by human neutrophil lysosomes and we hypothesized that the toxin acts as a kinase capable of either directly phosphorylating the neutrophil lysosomal Ca^{++} ATPase or indirectly activating it by phosphorylation of a regulatory intermediate.

Numerous phosphorylation studies on intact neutrophil lysosomes as well as on lysosomal membranes failed to reveal any effect of TT on protein phosphorylation or dephosphorylation.

We then asked the question whether TT acts on lysosomes, since our neutrophil "lysosomal" preparation is actually a microsomal one that consists of two intracellular organelle populations, lysosomes and mitochondria (1). To determine which organelle was affected by TT, we performed Ca^{++} uptake experiments using mitochondrial inhibitors.

As shown in Figure 1, either one or both of the mitochondrial inhibitors azide (5 mM) and atractyloside (100 uM) partially inhibited the ATP-dependent Ca^{++} uptake of PMN microsomes, indicating that a significant portion of Ca^{++} uptake by the preparation was due to mitochondria. The stimulatory effect of TT (5 ug/ml), however, was completely abolished by either one or both azide and atractyloside (Figure 1), strongly suggesting that TT exerts its effect on mitochondria.

Further evidence was provided by the use of the mitochondrial substrate, succinate. As shown in Figure 2, succinate (1 mM) increased the microsomal ATP-dependent Ca^{++} uptake, which was further increased by the presence of TT (5 ug/ml). Since TT stimulates the succinate-driven increase in ATP-dependent Ca^{++} uptake, and since only mitochondria can utilize succinate to energize Ca^{++} uptake, we concluded that TT acts on the mitochondria in our preparation.

To confirm this conclusion, we purified mitochondria from bovine adrenal cortex since previous work demonstrated that adrenal cells could be intoxicated by intracellular injection of TT. Highly purified preparations of bovine adrenal cortex mitochondria were prepared according to well characterized methods (2-5). As illustrated in Figure 3, Ca^{++} uptake by adrenal cortex mitochondria in the presence of ATP (1 mM) was increased by increasing concentrations of TT. At 1 ug/ml TT, the ATP-dependent Ca^{++} uptake was significantly increased.

Our next step was to show that ATP-dependent Ca^{++} uptake by isolated mitochondria was inhibited by either one or both of the mitochondrial inhibitors azide and atractyloside. As shown in Figure 4, either one or both azide and atractyloside completely inhibited ATP-dependent Ca^{++} uptake activity by mitochondria in the presence or absence of TT (5 ug/ml).

We next investigated whether the effect of TT was supported by the nonhydrolyzable ATP analogue AMP-PNP (6). As shown in Figure 5, TT had no effect on mitochondrial Ca^{++} uptake in the presence of the ATP analogue, indicating a requirement for a hydrolyzable high energy phosphate bond.

Since we showed that the effect of TT on mitochondrial Ca^{++} uptake requires ATP, our next step was to investigate the mechanism by which it does so. The most tempting hypothesis was that TT acts as a kinase, phosphorylating a protein(s) that participates in Ca^{++} uptake by mitochondria, or, acting indirectly, phosphorylates a kinase, which in turn phosphorylates another protein. Since all known protein kinases require ATP concentrations in the micromolar range (7-10), the results shown in Figure 6 argue against TT having kinase activity. The studies demonstrated that

TT stimulated ATP-dependent Ca^{++} uptake by bovine adrenal cortex mitochondria only at 1 mM and at 0.5 mM ATP. There was little activity of TT at 100 uM ATP and no effect at 10 uM ATP, indicating that TT is not a typical protein kinase.

We next attempted to show a specificity for the stimulatory effect of tetanus toxin (TT) on ATP-dependent Ca^{++} uptake by mitochondria. To our surprise, boiled toxin, which is biologically inactive, showed the same stimulatory effect. Also, TT dialyzed against the buffer used for the Ca^{++} uptake experiments showed no stimulatory effect on mitochondrial Ca^{++} uptake, at least by bovine adrenal mitochondria. A possible explanation is that the active factor is inorganic phosphate (P_i), which is abundant in our TT stock preparation, since its buffer is phosphate buffered saline (PBS), and since P_i is known to activate mitochondrial Ca^{++} uptake. When we measured the P_i concentration of our TT stock preparation, it was found to be 3 mM. We then tested various concentrations of P_i on mitochondrial ATP-dependent Ca^{++} uptake and found that 100 uM showed a stimulatory effect and 10 uM had no effect. According to our calculations (from the dilutions of the TT stock we are using) and to the above P_i measurement, the final concentrations of P_i in our experiments are always below 30 uM, and therefore should not have a notable effect on ATP-dependent Ca^{++} uptake. Furthermore, serial dilutions of PBS, equivalent to the dilutions of the TT stock we are using, were compared to the equivalent dilutions of TT. The PBS dilutions increased ATP-dependent Ca^{++} uptake, as was expected (since PBS contains P_i), but the increases were small compared to the corresponding increases induced by the TT preparation. These results indicated that the observed effect of TT is probably not attributable to the contamination of our TT preparation by phosphate salts.

After the results of the experiments described in the previous paragraph, we realized that we had to show some specificity for the TT effect, and we performed two groups of experiments.

In the first one, we applied our TT preparation to a TT-specific ganglioside (GT_b) column. We then tested the material eluted from this column. Although there was no TT detectable by protein gel electrophoresis, this material showed the same activity as control TT preparations in increasing ATP-dependent Ca^{++} uptake by either human neutrophil microsomes or bovine adrenal cortex mitochondria.

In the second group of experiments, we eliminated the toxin from our preparation by immunoabsorption using protein A sepharose beads and the anti-TT monoclonal antibodies provided by Dr. William Habig. Unfortunately, although all of the toxin present in our preparation was immunoabsorbed as confirmed by protein gel electrophoresis, the material still had the same activity as control TT preparations in augmenting ATP-dependent Ca^{++} uptake by either human neutrophil lysosomes or bovine adrenal cortex mitochondria.

The last two groups of experiments confirmed that the effect of TT we have been observing was artefactual. We suspect that this was due to contamination of our TT preparation probably by phosphate salts. We do not wish to further investigate what factor caused the increased ATP-dependent Ca^{++} uptake, since we know that it definitely is not tetanus toxin.

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FIGURE 1

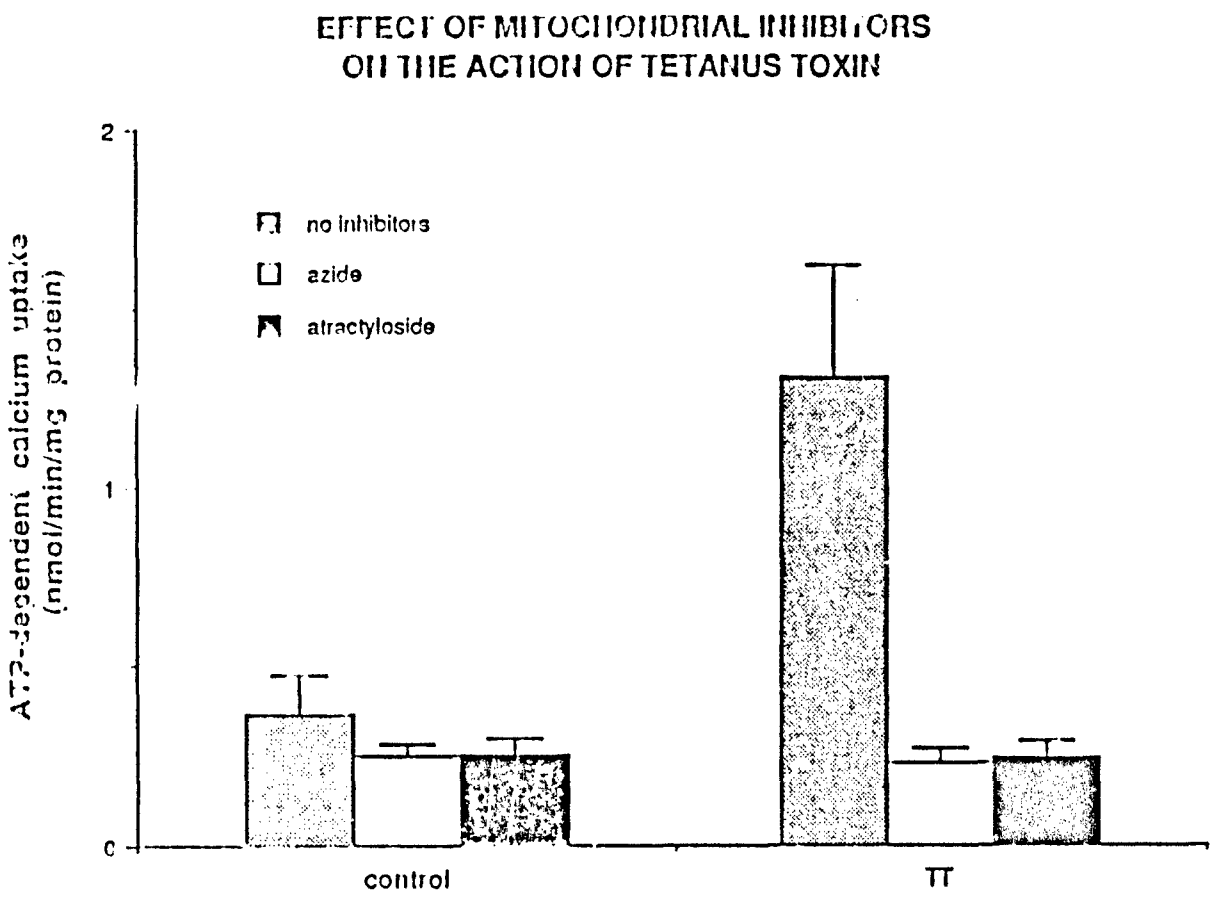

EFFECT OF MITOCHONDRIAL INHIBITORS
ON THE ACTION OF TETANUS TOXIN
ATP-dependent calcium uptake
(nmol/min/mg protein)
2
1
0
no inhibitors
azide
atractyloside
control
TT

FIGURE 2

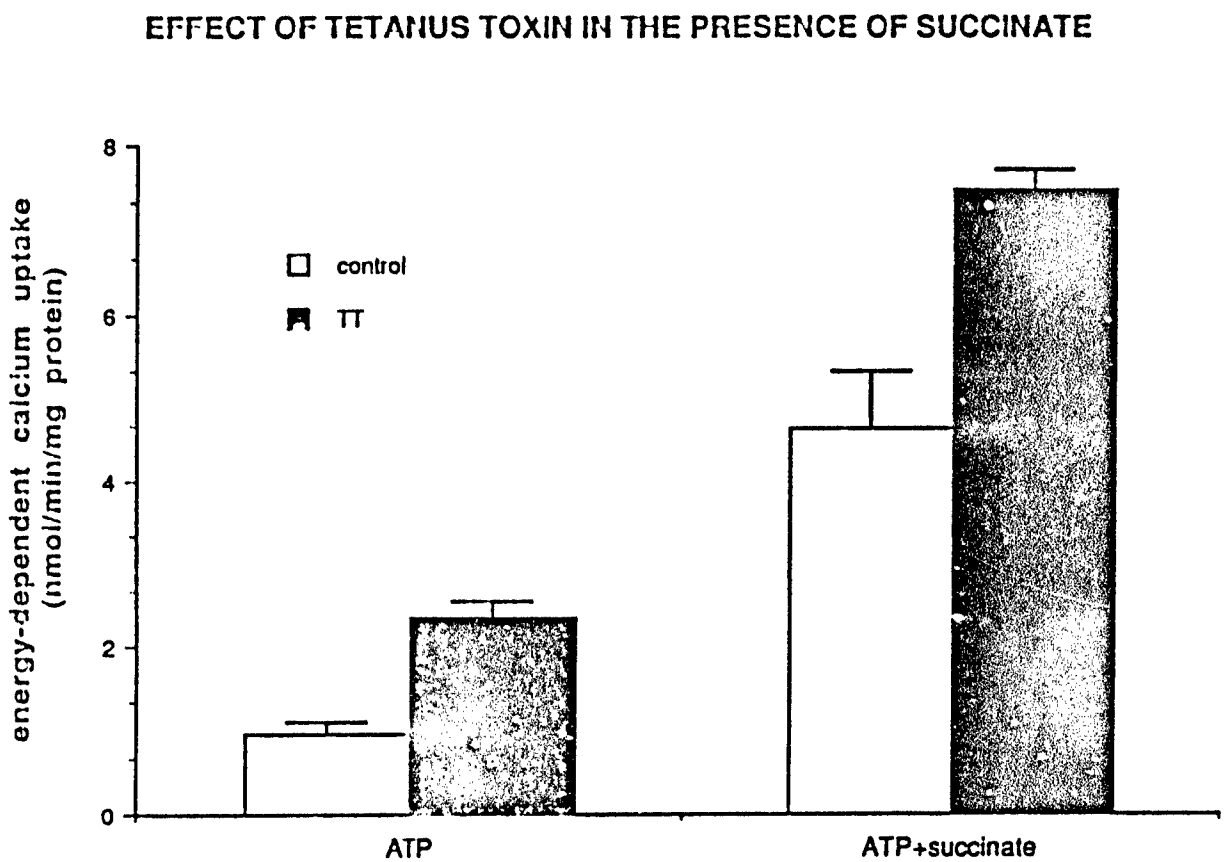

EFFECT OF TETANUS TOXIN IN THE PRESENCE OF SUCCINATE
energy-dependent calcium uptake
(nmol/min/mg protein)
8
6
4
2
0
control
TT
ATP
ATP+succinate

FIGURE 3

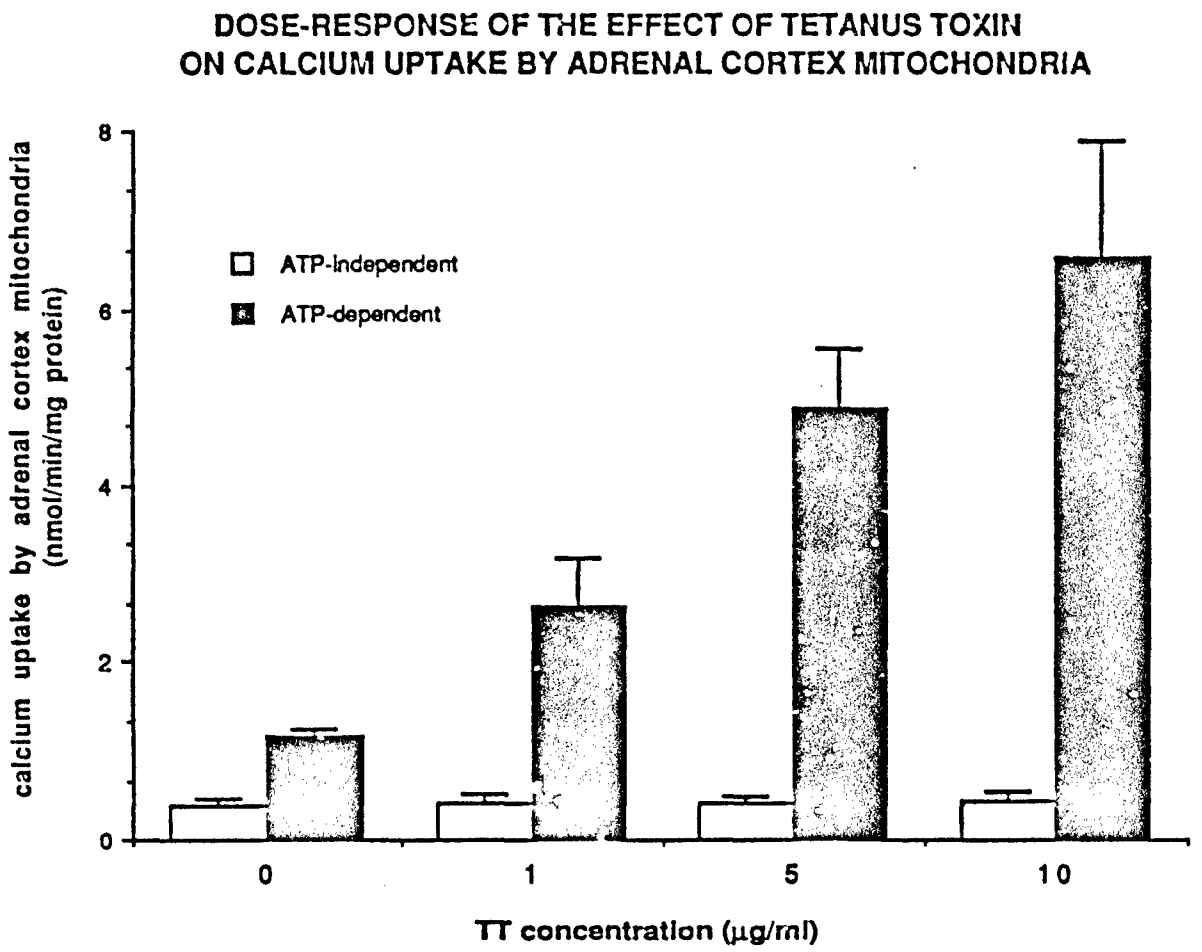

DOSE-RESPONSE OF THE EFFECT OF TETANUS TOXIN
ON CALCIUM UPTAKE BY ADRENAL CORTEX MITOCHONDRIA
calcium uptake by adrenal cortex mitochondria
(nmol/min/mg protein)
8
6
4
2
0
ATP-Independent
ATP-dependent
0
1
5
10
TT concentration (µg/ml)

FIGURE 4

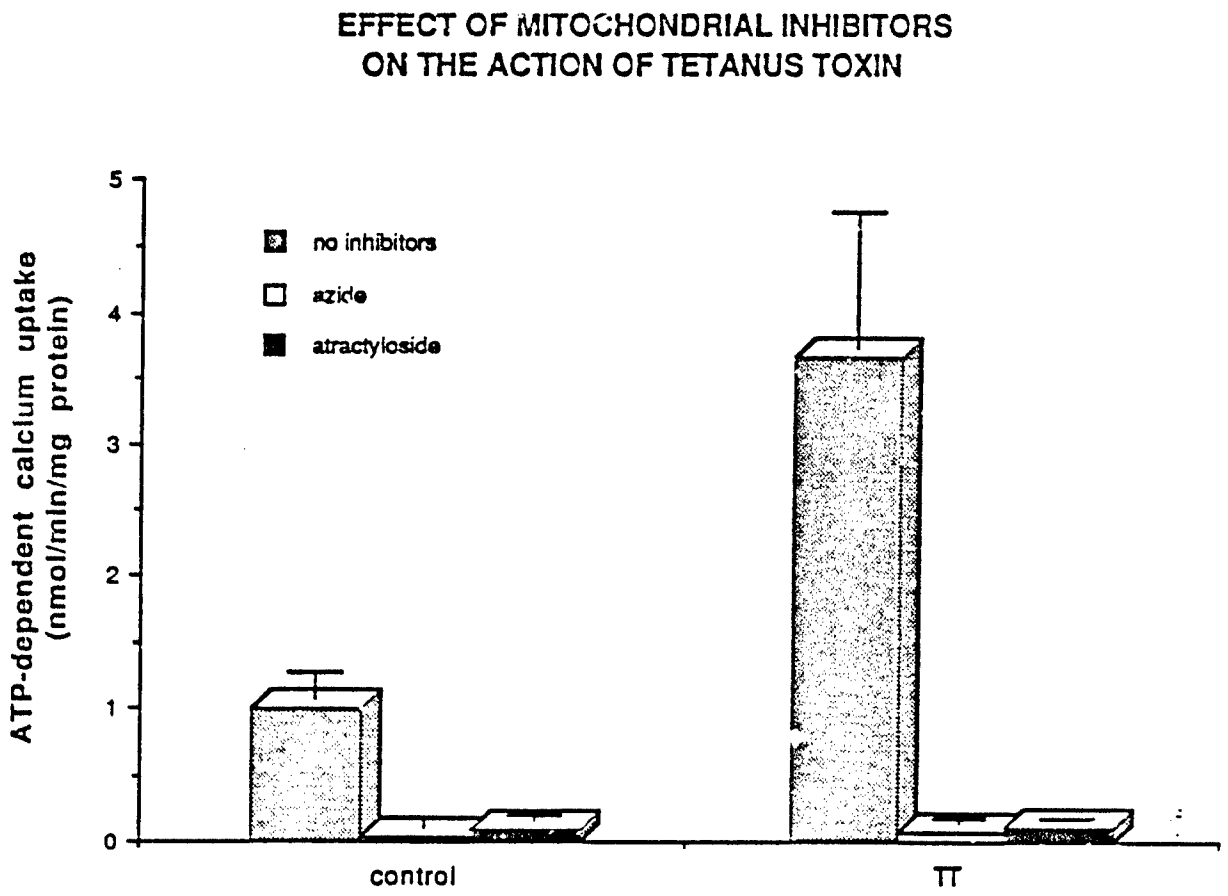

EFFECT OF MITOCHONDRIAL INHIBITORS
ON THE ACTION OF TETANUS TOXIN
ATP-dependent calcium uptake
(nmol/mln/mg protein)
5
4
3
2
1
0
no inhibitors
azide
atractyloside
control
TT

FIGURE 5

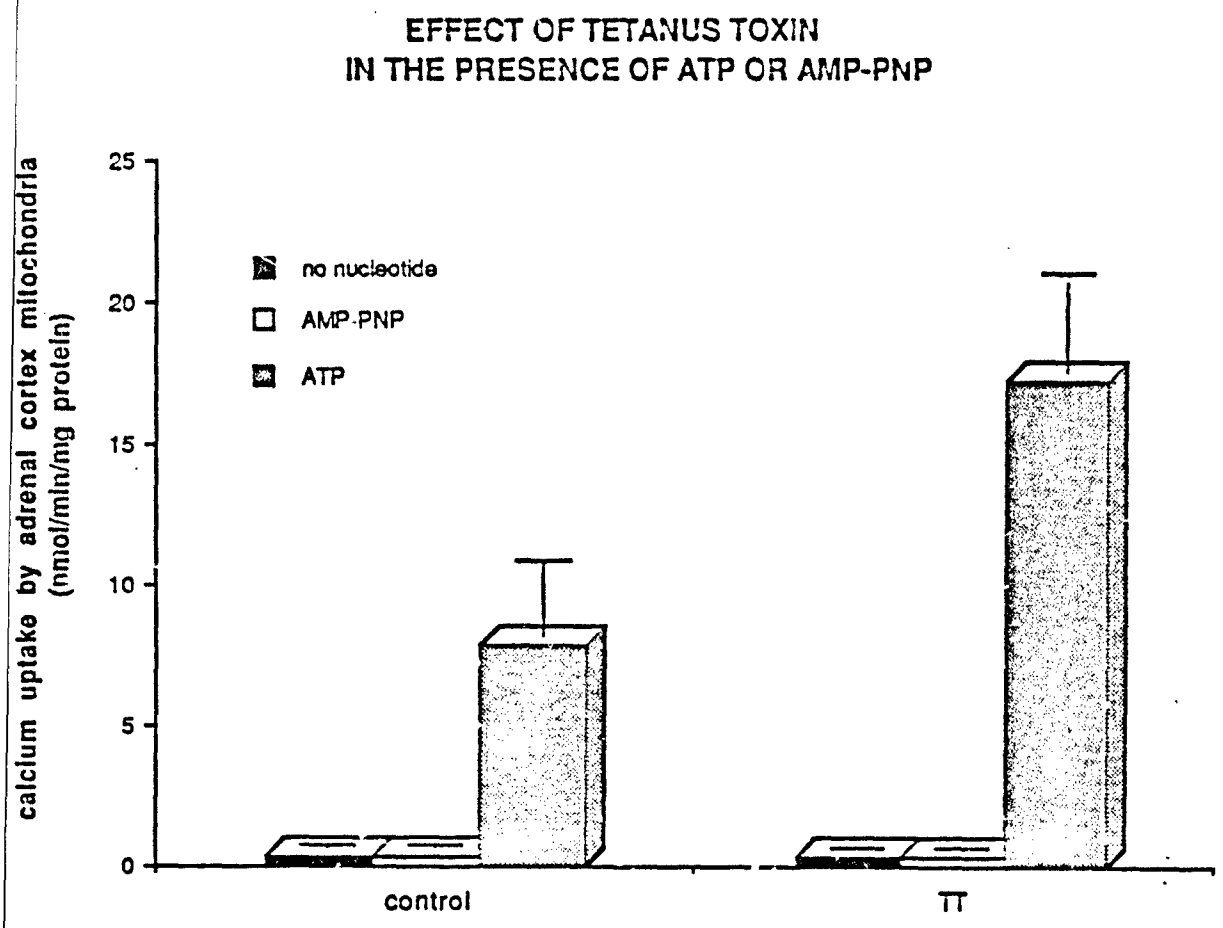
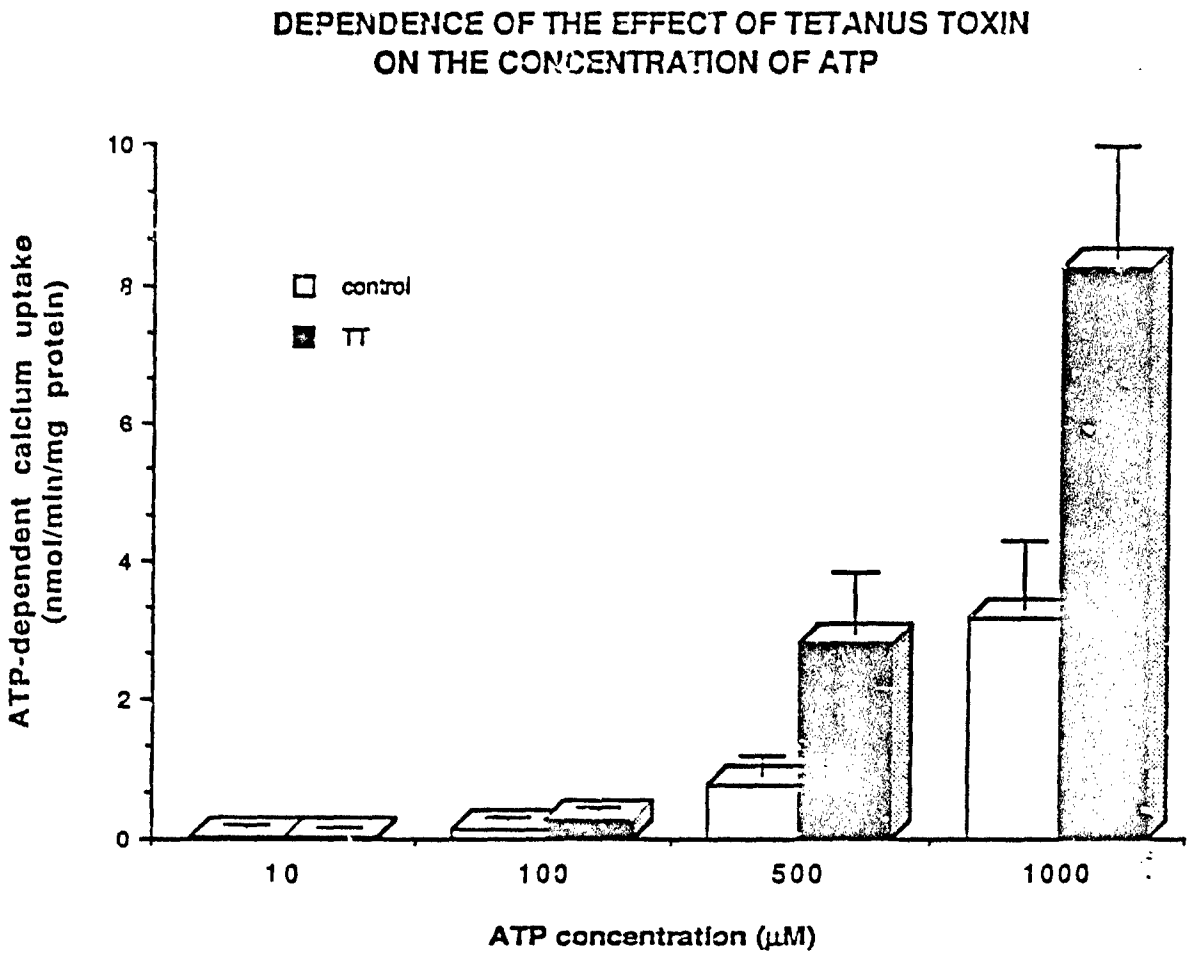

EFFECT OF TETANUS TOXIN
IN THE PRESENCE OF ATP OR AMP-PNP
calcium uptake by adrenal cortex mitochondria
(nmol/min/mg protein)
25
20
15
10
5
0
no nucleotide
AMP-PNP
ATP
control
TT

FIGURE 6


DEPENDENCE OF THE EFFECT OF TETANUS TOXIN
ON THE CONCENTRATION OF ATP
ATP-dependent calcium uptake
(nmol/min/mg protein)
control
TT
0
2
4
6
8
10
10
100
500
1000
ATP concentration (µM)